TITLE OF THE INVENTION

Low Temperature Expression Chitinase cDNAs and Method for Isolating the Same

BACKGROUND OF THE INVENTION

The present invention relates to chitinase cDNAs and to a method for their isolation, and more specifically it relates to chitinase cDNAs having a function of conferring plant disease resistance under low temperature, and to a method of isolating the chitinase cDNAs.

In the northern regions, overwintering crops such as barley, forage grasses and wheat must survive subzero temperature (0 ℃ or below 0 ℃) and a long-lasting snow cover condition (0 ℃ in darkness). However, overwintering crops in such environment are often attacked by snow molds which are a diverse group of psychrophilic parasitic fungi. This biotic stress greatly limits yields and quality of biennial or perennial crops, in the same manner as a low temperature stress will do in the northern region with snow accumulation.

In current winter wheat cultivation, it is necessary to apply a broad-spectrum fungicides before a continuous snow cover for protecting the plant from snow molds infection.

However, it has taken high cost and it has been proved difficult to apply the fungicide at the effective time, because of unstable nature of the start of a snow cover every year.

In view of the above, it has been desired to raise a plant variety having a high disease resistance under low temperature environment.

Nevertheless, up till now, when using several conventional breeding methods each based on cross-breeding, it has not been possible to raise superior varieties with high resistance, and a long time (many years) is required for raising superior varieties. For this reason, there has been a strong demand for variety improvement by more effective methods such as gene engineering methods.

As a result of repeated diligent research over years aimed at solving the problems described above, the inventors of the present invention have arrived at the following conclusion. Specifically, it has been found that plant disease resistance under low temperature environment is induced by cold acclimation that occurs under a low temperature from autumn through winter (hereunder referred to as "hardening") and that expression of the three chitinase cDNAs of the invention described hereunder are found during this hardening, with the translation product conferring plant disease resistance through digestion of chitin, one of the major components of fungus cell wall.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide chitinase cDNAs that encode proteins having enzymatic

function in low temperature environments and that when introduced into plants confer plant disease resistance.

It is another object of the invention to provide a method for isolation of chitinase cDNAs that encode proteins having enzymatic function in low temperature environments and that when introduced into plants confer plant disease resistance.

According to one aspect of the present invention, there is provided a winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 1 in Fig. 1. In detail, said cDNA comprises 771 nucleotides/256 amino acids and has 98% identity (on amino acid sequence level) with barley-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to another aspect of the present invention, there is provided another winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 2 in Fig. 2. In detail, said cDNA comprises 972 nucleotides/323 amino acids and has 68% identity (on amino acid sequence level) with rye-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant

disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to a further aspect of the present invention, there is provided a further winter wheat-derived chitinase cDNA, characterized in that said cDNA has a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 3 in Fig. 3. In detail, said cDNA comprises 960 nucleotides/319 amino acids and has 95% identity (on amino acid sequence level) with spring wheat-derived chitinase cDNA. In more detail, said cDNA encodes a protein with chitinase activity in low temperature environment and confers plant disease resistance by digestion of chitin, one of the major components of fungus cell wall.

According to a still further aspect of the present invention, there is provided a method of isolating a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 1 in Fig. 1, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 2 in Fig. 2, a winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to an amino acid sequence listed as SEQ. ID. No. 3 in Fig. 3, said method comprising the steps of: extracting mRNA from winter wheat variety PI173438 (having high snow molds resistance) that has undergone a sufficient hardening process; preparing cDNA and a cDNA library based on said mRNA; analyzing

nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genebank/DDBJDNA Databank; designing a pair of chitinase cDNA-specific degenerated primers with reference to highly conserved nucleotide sequence portions of the plant-derived chitinase cDNAs; conducting PCR (polymerase chain reaction) using a pair of chitinase cDNA-specific degenerated primers and using said cDNA as a template, thereby amplifying fragments of chitinase cDNAs and obtaining amplified DNA fragments; and using said amplified DNA fragments as probes for screening said cDNA library by a hybridization assay, to isolate recombinant plaques containing full length of cDNA.

In particular, one of the pair of chitinase cDNA-specific degenerated primers has the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-G-C (SEQ. ID. No. 4),

and the other has the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G (SEQ. ID. No. 5).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an amino acid sequence of SEQ. ID No. 1.

Fig. 2 shows an amino acid sequence of SEQ. ID No. 2.

Fig. 3 shows an amino acid sequence of SEQ. ID No. 3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The cDNAs of the present invention are chitinase cDNAs capable of expressing under a low temperature condition.

The method for isolating the cDNAs of the present invention may be carried out in the following manner.

Specifically, mRNA is extracted from winter wheat PI173438 (having high snow molds resistance) that has undergone a hardening process (low temperature acclimation) under natural conditions in Sapporo City, Japan until November 22. This mRNA is then used to prepare cDNA and a cDNA library.

Next, nucleotide sequences of a number of plant-derived chitinase cDNAs which have all been published by EMBL/Genebank/DDBJDNA Databank are closely analyzed, and a pair of chitinase cDNA-specific degenerated primers are designed with reference to highly conserved nucleotide sequence portions.

The pair of designed chitinase cDNA-specific degenerated primers are used in a PCR (polymerase chain reaction) using the above-mentioned cDNA as the template for amplifying the expected chitinase cDNA fragments (all are approximately 400 bp), and the amplified fragments are isolated.

The amplified fragments are used as probes for screening the cDNA library by a hybridization assay, to isolate recombinant plaques containing full length of cDNA. The nucleotide sequences of the isolated plaques were analyzed and demonstrated to be three different chitinase cDNAs which are

three kinds of chitinase cDNA fragments, all are novel in plants.

An example of the method for isolating the cDNAs of the present invention was carried out in the following steps 1) - 3).

1) Preparation of cDNA and cDNA library from snow molds resistant winter wheat variety PI173438

mRNA was extracted by a common method from the crown portion of winter wheat (Triticum astivum L.) PI173438 (having high snow molds resistance) that had been seeded in a container in late September and had then undergone a hardening process under natural conditions until November 22. A portion (5 μg) of the obtained mRNA was used to synthesize cDNA utilizing a cDNA Synthesis Kit (STRATAGENE Co.). After attaching adaptors to both ends of the cDNA, it was incorporated into a ZAP Expression Vector (STRATAGENE Co.), thereby obtaining a cDNA library of approximately 6 x 10^6 pfu.

2) PCR using a pair of cDNA-specific degenerated primers and using the cDNA as a template

One of the pair of chitinase cDNA-specific degenerated primers, having the following nucleotide sequence:

(Forward): 5' C-A-C-G-A-G-A-C-C-A-C-N-G-G-C-G-G-N-T-G-G-G-C (SEQ. ID. No. 4),

the other chitinase cDNA-specific degenerated primer, having the following nucleotide sequence:

(Reverse): 5' A-C-N-A-A-T-A-T-C-A-T-C-A-A-C-G-G-C-G-G

(SEQ. ID. No. 5).

which were synthesized based on highly conserved regions of the nucleotide sequences of known chitinase cDNAs (published by EMBL/Genebank/DDBJDNA Databank), were used in a PCR using the cDNA (synthesized in the manner described in the above) as the template.

The PCR was performed in a final volume of 50 μl. In detail, 1 μl of Taq DNA polymerase (5 units/μl) by Nippon Gene Co., 5 μl of 10 x PCR buffer (containing $MgCl_2$), 5 μl of dNTP solution (10 mM), 2 μl of each primer (12 μM) and about 10 ng of the cDNA synthesized in the above, were mixed and then brought to a total of 50 μl with distilled water. The PCR conditions and number of reaction cycles are shown in Table 1 below.

Table 1

PCR condition and number of reaction cycles

Initial Denaturation	94° C	1 min	once
Denaturation Annealing Primer Extension	94° C 48° C 72° C	1 min 1 min 1 min	30 cycles
Final Extension	72° C	2 min	once

(In Table 1, "denaturation" refers to a reaction in which double-stranded DNA is melt into single strand and secondary structure is eliminated, "primer extension" refers to an synthesizing of the new complementary strand, and "30 cycles" means that three basic steps of denaturation-annealing-primer extension are repeated with 30 cycles.

As a result, DNA fragments (having expected length of approximately 400 bp) of chitinase cDNAs were amplified by the above PCR with the pair of chitinase cDNA-specific degenerated primer having nucleotide sequence of SEQ.ID No.4 and the primer with the nucleotide sequence of SEQ.ID No.5. Theses amplified DNA fragments were then isolated and subsequently sequenced using a DNA sequencer (Model 373S by ABI Co.) according to the conventional method. By comparing the

sequences with known chitinase, it were confirmed that novel chitinase cDNA fragments (having a high homology with known chitinase cDNA) were isolated.

3) Isolation and nucleotide sequencing of full length cDNAs encoding chitinase of the present invention

About $1x10^5$ recombinant plaques from the cDNA library obtained in the manner described in the above were subjected to a hybridization assay by using filters lifted with $1x10^5$ recombinant plaques, and using probes prepared by labeling (with ^{32}P) each novel chitinase cDNA fragment obtained in the above.

The hybridization reaction was carried out for 16 hours at 42° C, in a solution containing 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.5% SDS and 0.2 mg/ml salmon sperm DNA with ^{32}P-labeled probe.

The filters were then washed twice in a solution containing 2 x SSC and 0.1% SDS at 65 ℃ for 10 min. Afterwards, the filters were washed twice with another washing solution containing 0.1 x SSC and 0.1% SDS, at 65 ℃ for 15 min. Detection of each positive plaque binding to ^{32}P-labed probe was performed by exposing above washed filters to X-ray films.

About 45 positive recombinant plaques obtained in the above were subjected to nucleotide sequencing with DNA sequencer by ABI Co.

Analysis of the nucleotide sequences of these recombinant plaques revealed that novel chitinase cDNAs having nucleotide sequences corresponding to the amino acid sequences listed as SEQ. ID Nos. 1 - 3 in Figs. 1 - 3 had been isolated from winter wheat variety PI173438.

In fact, what were isolated were i) a novel winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to the amino acid sequence listed as SEQ. ID. No. 1 in Fig. 1, comprising 771 nucleotides/256 amino acids and having 98% identity (on amino acid sequence level) with barley-derived chitinase cDNA, ii) a novel winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to the amino acid sequence listed as SEQ. ID. No. 2 in Fig. 2, comprising 972 nucleotides/323 amino acids and having 68% identity (on amino acid sequence level) with rye-derived chitinase cDNA, iii) a novel winter wheat-derived chitinase cDNA having a nucleotide sequence corresponding to the amino acid sequence listed as SEQ. ID. No. 3 in Fig. 3, comprising 960 nucleotides/319 amino acids and having 95% identity (on amino acid sequence level) with spring wheat-derived chitinase cDNA.

Investigation of Enzymatic Activity

In order to investigate enzymatic activities of the novel chitinase cDNAs of the present invention, enzymatic reactions were conducted under the following conditions using

culture solutions containing novel proteins secreted by recombinant yeast (into which each novel chitinase cDNA of the present invention has been introduced).

[Enzymatic Reaction Condition]

Buffer solution (20 mM citric acid/phosphoric acid), pH 4.5

Final substrate concentration: 1% collidal chitin

Reaction temperature: 38 ℃, reaction time: 16 hours.

As a result, it was confirmed that the culture solutions containing novel proteins secreted by recombinant yeast (into which each novel chitinase cDNA of the present invention has been introduced) had a chitinase activity capable of producing a disaccharide (a chito-oligosaccharide) or a trisaccharide (another chito-oligosaccharide) from chitin polymer (serving as a substrate).

The nucleotide sequences of the novel cDNAs obtained in the present invention are listed in the following.

Nucleotide Sequence of cDNA corresponding to the

B Amino Acid Sequence Listed as SEQ. ID. No. 1 (SEQ ID NO:6)

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        10         20         30         40         50         60
ATGGCGAGGT TTGCTGCCCT CGCCGTGTGC GCCGCCGCGC TCCTGCTCGC CGTGGCGGCG

        70         80         90        100        110        120
GGGGGTGCCG CGGCGCAGGG CGTGGGCTCG GTCATCACGC GGTCGGTGTA CGCGAGCATG

       130        140        150        160        170        180
CTGCCCAACC GCGACAACTC GCTGTGCCCG GCCAGAGGGT TCTACACGTA CGACGCCTTC

       190        200        210        220        230        240
ATCGCCGCCG CCAACACCTT CCCGGGCTTC GGCACCACCG GCAGCGCCGA CGACATCAAG

       250        260        270        280        290        300
CGCGACCTCG CCGCCTTCTT CGGCCAGACC TCCCACGAGA CCACCGGAGG GACGAGAGGC

       310        320        330        340        350        360
GCTGCCGACC AGTTCCAGTG GGGCTACTGC TTCAAGGAAG AGATAAGCAA GGCCACGTCC

       370        380        390        400        410        420
CCACCATACT ATGGACGGGG ACCCATCCAA TTGACAGGGC GGTCCAACTA CGATCTTGCC

       430        440        450        460        470        480
GGGAGAGCGA TCGGGAAGGA CCTGGTGAGC AACCCAGACC TAGTGTCCAC GGACGCGGTG

       490        500        510        520        530        540
GTGTCCTTCA GGACGGCCAT GTGGTTCTGG ATGACGGCGC AGGGAAACAA GCCGTCGTGC

       550        560        570        580        590        600
CACAACGTCG CCCTACGCCG CTGGACGCCG ACGGCCGCCG ACACCGCTGC CGGCAGGGTA

       610        620        630        640        650        660
CCCGGATACG GAGTGATCAC CAATATCATC AACGGCGGGC TCGAGTGCGG AATGGGCCGG

       670        680        690        700        710        720
AACGACGCCA ACGTCGACCG CATCGGCTAC TACACGCGCT ACTGCGGCAT GCTCGGCACG

       730        740        750        760        770        780
GCCACCGGAG GCAACCTCGA CTGCTACACC CAGAGGAACT TCGCTAGCTA G.........
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Nucleotide Sequence of cDNA corresponding to the

B Amino Acid Sequence Listed as SEQ. ID. No. 2 (SEQ ID NO:7)

10	20	30	40	50	60
ATGTCCACGC	TGAGAGCGCG	GTGTGCGACG	GCCGTCCTGG	CCGTCGTCCT	GGCGGCGGCC
70	80	90	100	110	120
GCGGTCACGC	CGGCCACGGC	CGAGCAGTGC	GGCTCGCAAG	CCGGCGGCGC	CAAGTGCGCC
130	140	150	160	170	180
GACTGCCTGT	GCTGCAGCCA	GTTCGGGTTC	TGCGGCACCA	CCTCCGACTA	CTGCGGCCCC
190	200	210	220	230	240
CGCTGCCAGA	GCCAGTGCAC	TGGCTGCGGT	GGCGGCGGCG	GCGGGGTGGC	CTCCATCGTG
250	260	270	280	290	300
TCCAGGGACC	TCTTCGAGCG	GTTCCTGCTC	CATCGCAACG	ACGCAGCGTG	CCTGGCCCGC
310	320	330	340	350	360
GGGTTCTACA	CGTACGACGC	CTTCTTGGCC	GCCGCCGGCG	CGTTCCCGGC	CTTCGGCACC
370	380	390	400	410	420
ACCGGAGACC	TGGACACGCG	GAAGCGGGAG	GTGGCGGCCT	TCTTCGGCCA	GACCTCTCAC
430	440	450	460	470	480
GAGACCACCG	GCGGGTGGCC	CACCGCGCCC	GACGGCCCCT	TCTCATGGGG	CTACTGCTTC
490	500	510	520	530	540
AAGCAGGAGC	AGGGCTCGCC	GCCGAGCTAC	TGCGACCAGA	GCGCCGACTG	GCCGTGCGCA
550	560	570	580	590	600
CCCGGCAAGC	AGTACTATGG	CCGCGGCCCC	ATCCAGCTCA	CCCACAACTA	CAACTACGGA
610	620	630	640	650	660
CCGGCGGGCC	GCGCAATCGG	GGTGGACCTG	CTGAACAATC	CGGACCTGGT	GGCCACGGAC
670	680	690	700	710	720
CCGACAGTGG	CGTTCAAGAC	GGCGATATGG	TTCTGGATGA	CGACGCAGTC	CAACAAGCCG
730	740	750	760	770	780
TCGTGCCATG	ACGTGATCAC	GGGGCTGTGG	ACTCCGACGG	CCAGGGATAG	CGCAGCCGGA
790	800	810	820	830	840
CGGGTACCCG	GGTATGGTGT	CATCACCAAC	GTCATCAACG	GCGGGATCGA	ATGCGGCATG
850	860	870	880	890	900
GGGCAGAACG	ACAAGGTGGC	GGATCGGATC	GGGTTCTACA	AGCGCTATTG	TGACATTTTC
910	920	930	940	950	960
GGCATCGGCT	ACGGGAATAA	CCTCGACTGC	TACAACCAAT	TGTCGTTCAA	CGTTGGGCTC
970	980	990	1000	1010	1020
GCGGCACAGT	GA........	....	....	....	....

Nucleotide Sequence of cDNA Corresponding to the Amino Acid Sequence Listed as SEQ. ID. No. 3 (SEQ ID NO:8)

10	20	30	40	50	60
ATGAGAGGAG	TTGTGGTGGT	GGCCATGCTG	GCCGCGGCCT	TCGCCGTGTC	TGCGCACGCC
70	80	90	100	110	120
GAGCAATGCG	GCTCGCAGGC	CGGCGGGGCG	ACGTGCCCCA	ACTGCCTCTG	CTGCAGCAAG
130	140	150	160	170	180
TTCGGTTTCT	GCGGCACCAC	CTCCGACTAC	TGCGGCACCG	GCTGCCAGAG	CCAGTGCAAT
190	200	210	220	230	240
GGCTGCAGCG	GCGGCACCCC	GGTACCGGTA	CCGACCCCCT	CCGGCGGCGG	CGTCTCCTCC
250	260	270	280	290	300
ATTATCTCGC	AGTCGCTCTT	CGACCAGATG	CTGCTGCACC	GCAACGACGC	GGCGTGCCTG
310	320	330	340	350	360
GCCAAGGGGT	TCTACAACTA	CGGCGCCTTC	GTCGCCGCCG	CCAACTCGTT	CTCGGGCTTC
370	380	390	400	410	420
GCGACCACAG	GTAGCACCGA	CGTCAAGAAG	CGCGAGGTGG	CCGCGTTCCT	CGCTCAGACT
430	440	450	460	470	480
TCCCACGAGA	CGACCGGCGG	GTGGCCGACG	GCGCCCGACG	GCCCCTACTC	CTGGGGCTAC
490	500	510	520	530	540
TGCTTCAACC	AGGAGCGCGG	CGCCACCTCC	GACTACTGCA	CGCCGAGCTC	GCAGTGGCCA
550	560	570	580	590	600
TGTGCGCCGG	GCAAGAAGTA	CTTCGGGCGC	GGGCCCATCC	AGATCTCACA	CAACTACAAC
610	620	630	640	650	660
TACGGGCCGG	CGGGGCAGGC	CATCGGCACC	GACCTGCTCA	ACAACCCGGA	CCTTGTGGCG
670	680	690	700	710	720
TCGGACGCGA	CCGTGTCGTT	TAAGACGGCG	TTGTGGTTCT	GGATGACGCC	GCAATCACCC
730	740	750	760	770	780
AAGCCTTCGA	GCCACGACGT	GATCACGGGC	CGGTGGAGCC	CCTCGGGCGC	CGACCAGGCG
790	800	810	820	830	840
GCGGGGAGGG	TGCCTGGGTA	CGGTGTGATC	ACCAACATCA	TCAACGGTGG	GCTCGAGTGC
850	860	870	880	890	900
GGGCGCGGGC	AGGACGGCCG	TGTCGCCGAC	CGGATCGGGT	TCTACAAGCG	CTACTGCGAC
910	920	930	940	950	960
CTCCTTGGCG	TCAGCTACGG	TGACAACCTG	GACTGCTACA	ACCAAAGGCC	GTTCGCATAG
970	980	990	1000	1010	1020
....	....	....	....	....	....

The advantages of the present invention may be concluded as follows.

According to the present invention there are provided novel chitinase cDNAs in wheat that have different amino acid sequences from known chitinase cDNAs and confer high disease resistance in low temperature environment. Because the three chitinase cDNAs of the present invention are capable of digesting chitin at low temperature, the introduction of any one of these three different chitinase cDNAs into plants can confer plant disease resistance in low temperature environments, so that plant varieties can be provided with high resistance against psychrophilic plant pathogens such as snow molds.

While the presently preferred embodiments of the this invention have been shown and described above, it is to be understood that these disclosures are for the purpose of illustration and that various changes and modifications may be made without departing from the scope of the invention as set forth in the appended claims.